IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Shigeo SHIBATANI et al.

Appln. No. 10/566,157 Group Art Unit: 1638

Filed: 01/27/2006 Examiner: Brendan O BAGGOT

For: PLANT PRODUCING HYALURONIC ACID

DECLARATION

Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir :

I, Shigeo SHIBATANI, hereby declare:

1) That I am the first inventor of the instant invention; and

2) That the experiments given below were carried out under my general direction and supervision.

1. Hyaluronic Acid Production by Transformed Plant Cells with Hyaluronic Acid Synthase Genes from Different Origins

Transformation of cultured tobacco cells (BY-2) was performed according to An's method (An, 1985, Plant Physiol, 79, 568-570). Hyaluronic acid synthase (HAS) genes from mice (mHAS1), *Streptococcus pyogenes* (spHAS), chlorella virus Hirosaki strain (cvHAS-HI), and chlorella virus Kakunodate strain (cvHAS-KA) were used for the

transformation. Each gene was inserted into a pBI121 vector, which was introduced into *Agrobacterium tumefaciens* strain LBA4404 while pBI121 vector was used as a control (Mock). Thus obtained transformed *Agrobacterium* was used to infect BY-2 cells. Two to three weeks after infection, callused cells were transferred onto new plates for the selection of growing cells, which were then cultured continuously in 30 mL of modified LS media containing 100 mg/L kanamycin and 250 mg/L carbenicillin. The expression of each HAS gene in the transformed BY-2 cells was confirmed through RT-PCR method.

In vitro assay of hyaluronic acid synthase was performed as follows: crude membrane fractions prepared from the BY-2 cells were incubated with both UDP-GlcA and UDP-GlcNAc. Enzyme-linked immunosorbent assay (ELISA) using a hyaluronic acid plate called "Chugai" (Fujirebio, Inc.) was applied for the quantification of the hyaluronic acid concentrations. A significant amount of hyaluronic acid was detected in the reaction mixtures containing the membrane preparations derived from the transformed cells expressing cvHAS. No detectable hyaluronic acid synthase activities were observed in the membrane preparations derived from the control (wild-type and mocks) or transformed cells expressing mHAS1 or spHAS (Table I).

Table 1 *In vitro* HAS activity of BY-2 cells.

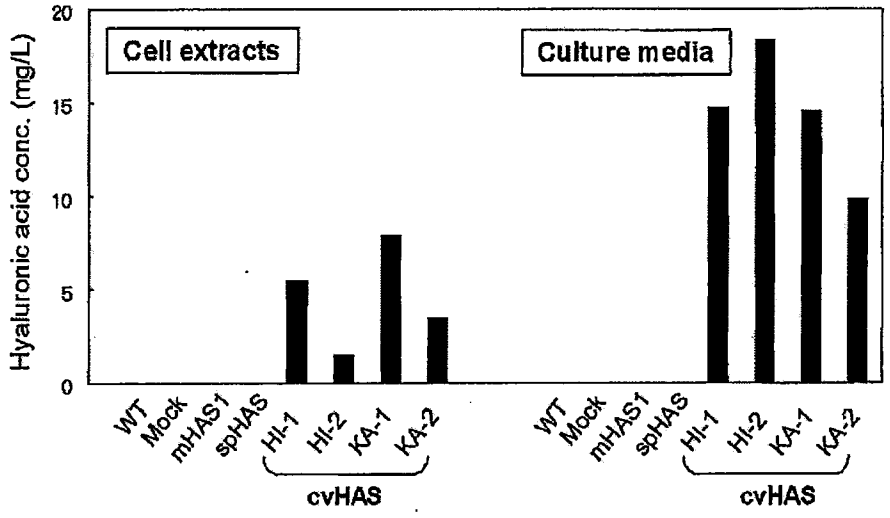
	HAS activity of crude membrane fraction (ng-HA/h/mg-protein)
Wild	N. D.
Mock	N. D.
mHAS1	N. D.
spHAS	N. D.
cvHAS,HI-1	4.5
cvHAS,HI-2	4.9
cvHAS,KA-1	6.5
cvHAS,KA-2	9.1

N.D.: not detected

The HAS-expressing cells were collected, disrupted and centrifuged at 1,000 rpm for 20 minutes, and the supernatant was collected as a crude extract of the transformants. The amount of hyaluronic acid in the cell extract and the culture media was measured using above method. The results are shown in Figure A below.

Fig. A

Production of hyaluronic acid in transgenic BY-2 cells expressing HAS



As shown in Figure A, significant amounts of hyaluronic acid were detected from the cell extracts and culture media for the transformed BY-2 cells expressing cvHAS-HI (HI-1 and HI-2), and cvHAS-KA (KA-1 and KA-2). In contrast, no hyaluronic acid was detected in either cell extracts or media for untransformed BY-2 cells (WT), BY-2 cells transformed with pBI121 vector (mock), mHAS1-expressing BY-2 cells (mHAS1) and spHAS-expressing BY-2 cells. These results clearly show that plant cells surprisingly acquire the ability to produce hyaluronic acid only when transformed with HAS gene from chlorella virus as opposed

to when transformed with HAS genes from other origins, such as mice and microorganisms.

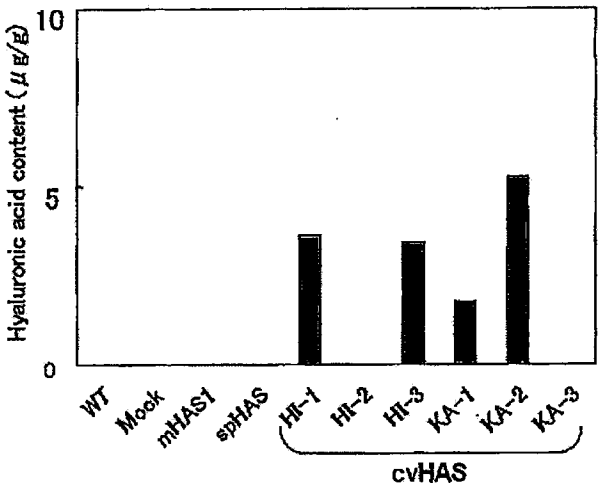
2. Hyaluronic Acid Production in a Transformed Plant with HAS Genes from Different Origins

Transformation of tobacco (*Nicotiana tabacum* SR-1) was performed according to a leaf disc method using *Agrobacterium* (Horsch et al., 1985, Science, 227, 129) . Hyaluronic acid synthase (HAS) genes from mice (mHAS), *Streptococcus pyogenes* (spHAS), chlorella virus Hirosaki strain (cvHAS-HI), and chlorella virus Kakunodate strain (cvHAS-KA) were used to obtain transformed *Agrobacterium* for infecting leaf discs of tobacco. Three weeks after the onset of inducing callus, morphologically normal shoots were selected, and transferred into rooting medium (MS inorganic salt, 3% sucrose, B5 vitamin and 0.3% gellan gum, pH 5.7) containing kanamycin (100 mg/L) and Claforan (250 mg/L) to induce rooting under the condition of 16 hour light at 25°C. After 2 weeks, shoots taking roots were transferred to fresh rooting medium, and multiple lines with growing stems and leaves were obtained. The expression of each HAS gene in the transformed tobacco lines was confirmed through RT-PCR method. Leaves (about 100 mg) from the transformed tobacco for which gene expression was confirmed were transferred to a 2 mL tube and suspended in

200 μL of buffer (containing 20 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA and 10 mM 2-ME), and 400 mg of zirconia beads (diameter 2 mm) were added thereto. The tobacco leaves were pulverized by shaking and agitating the tube using Bead Smash (Wakenyaku, BS-12) (2,500 rpm, 5 minutes). A solution after the pulverization was centrifuged (15,000 rpm, 10 minutes), and the supernatant was collected as a crude extract. The amount of hyaluronic acid in the extract was measured using a hyaluronic plate called "Chugai" (Fujirebio, Inc.). The results are shown in Figure B below.

Fig. B

Production of Hyaluronic Acid in Transgenic Tobacco Expressing HAS



As shown in Figure B, significant amounts of hyaluronic acid were detected in the extracts from the transformed

tobacco expressing cvHAS-HI (HI-1 and HI-3), and cvHAS-KA (KA-1 and KA-2). In contrast, no hyaluronic acid was detected in either extracts or media for non-infected tobacco (WT), tobacco transformed with pBI121 vector (control), mHAS1-expressing tobacco (mHAS1) and spHAS-expressing tobacco. These results clearly show that a plant surprisingly acquires the ability to produce hyaluronic acid only when transformed with HAS gene from chlorella virus as opposed to when transformed with HAS genes from other origins, such as mice and microorganisms.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: December 15, 2008

Shigeo Shibatani

Shigeo SHIBATANI